RAPTOR: A PORTABLE BIOSENSOR UPGRADED FOR RELIABILITY AND SENSITIVITY

George P. Anderson
Naval Research Laboratory
Center for Bio/Molecular Science and Engineering
Washington, D.C. 20375

ABSTRACT

The RAPTOR is a commercial, portable (5.6 kg) automated fiber optic biosensor able to detect up to four biological threat agents simultaneously. It performs rapid (5 to 13 minute) fluorescent sandwich immunoassays on the surface of four short polystyrene optical probes. The optical waveguide can be reused up to forty times, or until a positive result is obtained, reducing the logistical burden for field operations. The newest version of the RAPTOR uses the proven reliability of peristaltic pumps for its fluidics, which has greatly increased its mean time to failure. On the biochemical side, the use of Alexa Fluor 647 to label tracer antibodies and immobilization of biotinylated capture antibodies on avidin-coated waveguides have improved signal generation by a factor of 5. In combination with improved optics, the new RAPTOR has improved limits of detection by a log order. Recently, we demonstrated the capability of the device to test for eight targets by combining multiple antibodies on each waveguide. These improvements make the RAPTOR ready for use wherever rapid on-site biodetection is required.

INTRODUCTION

RAPTOR is a portable, 4-channel fluorescence-based immunoassay system (Figure 1), which has been developed by Research International in conjunction with the Naval Research Laboratory, where early work on fiber optic sensors occurred.[1-3] Previous models of the RAPTOR have been described[4-6], but with major system improvements implemented, which include custom peristaltic pumps to provide precise control over liquid movement, improved optical waveguides, and an optimized antibody attachment procedure, a review of its current capabilities is appropriate.

RAPTOR automatically performs multi-step fluoroimmunoassays, during which it simultaneously measures the signal from each of the system's four optical fibers. The four optical fibers are tested in parallel, allowing a sample to be analyzed for four different analytes. The fibers are mounted in a disposable assay cartridge, referred to as a "coupon". When the compartment door on the top of the device is closed, all of the fluidics connections to the coupon are made and the optics aligned. Thus, analyses may begin immediately since further manual manipulations are not required.

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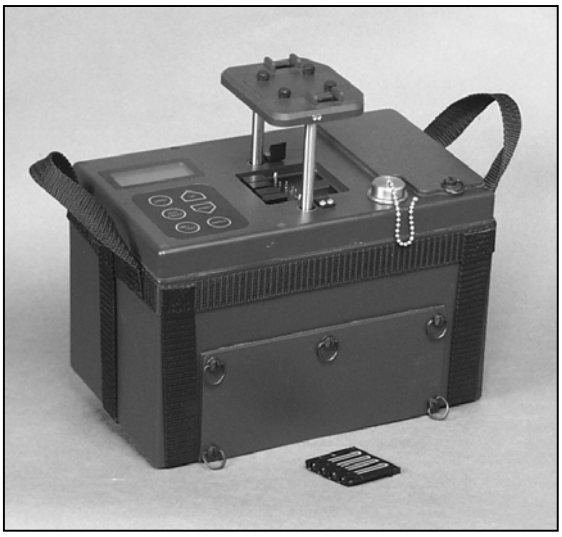


Figure 1. Field portable RAPTOR system used to detect biological agents

Each coupon has four individual flow channels corresponding to one optical fiber. Wash buffer, liquid sample (analyte), or air is pulled through each of the four chambers from a common source using a four-channel peristaltic pump. Tracer antibodies, held in separate reservoir vials, are pushed into each channel from the opposite direction using a separate four-channel pump. Thus, while all four fibers are exposed to the same analyte, each one may be separately interrogated using a different tracer antibody. This is a new feature of the current RAPTOR design; the previous design utilized a single tracer (or tracer cocktail) to interrogate all four fibers.

Although the RAPTOR's fluidics have been completely reconfigured, retained is the advantage of replacing the coupon only after a positive test, a valuable attribute in situations when a low incidence of positive samples is expected. The RAPTOR is capable of completing up to forty negative cycles without measurably impacting its limit of detection.[7-8] Thus, the ability to run sequential analyses without changing tracer antibodies or coupons significantly reduces the logistical burden over an instrument utilizing a disposable, single-use substrate. Examples of the aforementioned improvements are detailed below.

EXPERIMENTAL METHODS

BUFFERS AND REAGENTS

Wash buffer used for the RAPTOR was 8.3 mM phosphate buffer pH 7.2 + 0.05 % TritonX-100 (v/v) + 0.02 % sodium azide (w/v). Avidin, NeutrAvidin and NHS-LC-Biotin were purchased from Pierce (Rockland, IL). The anti-*F. tularensis* monoclonal antibody T-14 IgG was the kind gift of Dr. Peter Sveshnikov, from the Russian Research Center for Molecular Diagnostics and Therapy. The *F. tularensis* antigen (killed vaccine strain), affinity-purified rabbit anti-*B. anthracis* IgG, and rabbit anti- *B. globigii* were all generously provided by Naval Medical Research Center (Silver Sping, MD). The killed *B. anthracis* (Sterne strain) and *B. globigii* spores were provided by Dugway Proving Grounds (Dugway, UT).

PREPARATION OF BIOTINYLATED AND FLUORESCENTLY LABELED ANTIBODY

All biotinylated capture antibodies were prepared by reacting the antibodies with biotin-LC-NHS ester at a 1-to-5 molar ratio in 0.05 M sodium tetraborate, 0.04 M NaCl, pH 9.0, for 30 minutes. Unincorporated biotin was separated from the biotinylated antibodies by gel filtration purification using a Bio-Gel P10 column (Bio-Rad, Hercules CA).

The tracer antibodies were labeled with either Alexa Fluor 647 (Molecular Probes, Eugene, OR) or Cy5-bisfunctional dye (Amersham Biosciences, Piscataway, NJ); these fluorophores have essentially identical excitation and emission spectra. Antibodies labeled with Alexa Fluor were incubated with the reactive dye (31 μg dye per 0.33 mg protein) at pH 9 for 1 hr. Antibodies labeled with Cy5 were incubated with Cy5-NHS ester bisfunctional dye (1 packet per 3 mg IgG) at pH 8.5-9.0 for 30 minutes. Borate buffer (0.05M, 0.04M NaCl) was used for all reactions. After the appropriate incubation with activated fluorophore, Cy5- and Alexa Fluor-labeled proteins were separated from unincorporated dye by gel filtration using a Bio-Gel P10 column.

WAVEGUIDE / COUPON PREPARATIONS

Prior to immobilization of capture antibodies, the distal end of each waveguide was painted black to prevent any reflection of the excitation light. The waveguides were incubated overnight at 4^{o}C in 100 μg/mL NeutrAvidin (0.1 M sodium carbonate, pH 9.6). The avidin-coated fibers were then rinsed twice in Milli-Pore water and incubated in biotinylated capture antibody solutions (in phosphate buffered saline, PBS) for at least 2 hr at 4^{o}C. For assays using only a single capture antibody per waveguide, the concentration of biotinylated capture antibody in the solution was 100 μg/mL. When waveguides were coated with a mixture of three capture antibodies, the concentration of each antibody in solution was 33 μg/mL, giving a *total* IgG concentration of 100 μg/mL. This procedure resulted in essentially irreversible immobilization of capture antibodies onto the waveguide via avidin-biotin interaction.

Following treatment with capture antibodies, the probes were loaded directly into the coupon and glued using Norland 77 UV curing adhesive (Cranbury, NJ), creating a watertight seal. The coupon was then labeled to indicate the order of the optical fibers and sealed using a self-adhesive membrane.

ASSAY PROCEDURE

RAPTOR (Figure 1) is equipped with the optics, electronic, and fluidic components to perform sample analysis with minimal user interface. A small keypad and liquid crystal display (LCD) allow the user to perform assays with a single pushbutton. After the buffer and waste reservoirs are connected to the appropriate inlet and outlet, the four tracer reagent vials are placed in a specialized holder and are attached to their fluidics connectors on the instrument. The holder for the tracer reagents contains a calcium chloride hexahydrate solution that undergoes a phase change at approximately 28°C; therefore, this canister keeps the tracer reagents at or near room temperature for several hours even when operating in hot environments; however, the coupon is uncooled and thus, the biochemical assays are performed at ambient temperature.

After insertion and identification of the coupon by the unit, the assay is initiated by pressing the "Run Assay" button. First, the RAPTOR automatically performs a five-minute baseline analysis prior to testing the first sample. This analysis establishes a background by running an abbreviated assay using a buffer blank. This baseline accounts for any non-specific adsorption by the fluorescently labeled tracer antibodies. If the baseline is determined to be within acceptable limits, the unit will then ask the user to inject the first sample; subsequent samples are performed without additional baselines. For the assays

described here, an additional blank (1 mL wash buffer) was performed before analysis of any samples containing antigen.

The "sandwich" assay format used by the RAPTOR to detect biological agents is illustrated in Figure 2. Sample is first flowed into all waveguide chambers and incubated for seven minutes. After excess sample is flushed to waste, the waveguides are rinsed with wash buffer to remove all unbound antigen. The fluorescently labeled tracer antibodies (or antibody mixtures) are then flowed through the channels of the coupon and allowed to interact with the waveguides for 90 seconds; during this step, each waveguide is incubated with a different tracer antibody or cocktail. If target antigen is bound to the surface of the waveguide, the tracer antibody will bind to the captured analyte, forming a fluorescent complex on the surface of the waveguide; this fluorescent "sandwich" can be detected and quantified by the RAPTOR. Unbound tracers are then pumped back into their respective reservoir vials and the fibers are rinsed again with buffer.

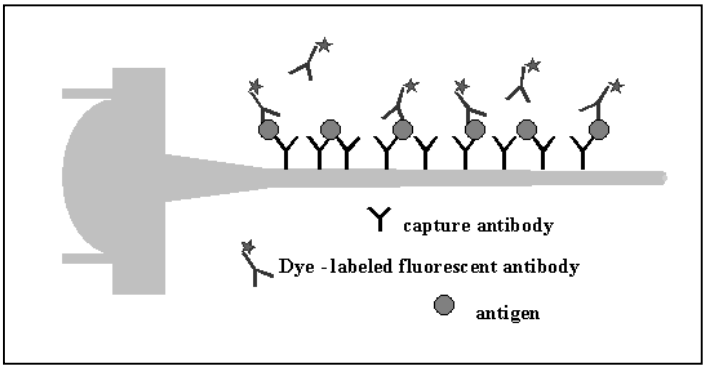


Figure 2. Diagram illustrating the sandwich assay used on the surface of the optical fiber in the RAPTOR (not to scale). The antibodies immobilized on the surface of the probe capture antigen in the first step. After washing away unbound material, a fluorescently labeled antibody is introduced which binds to the captured antigen, forming a fluorescent complex on the surface which is detected.

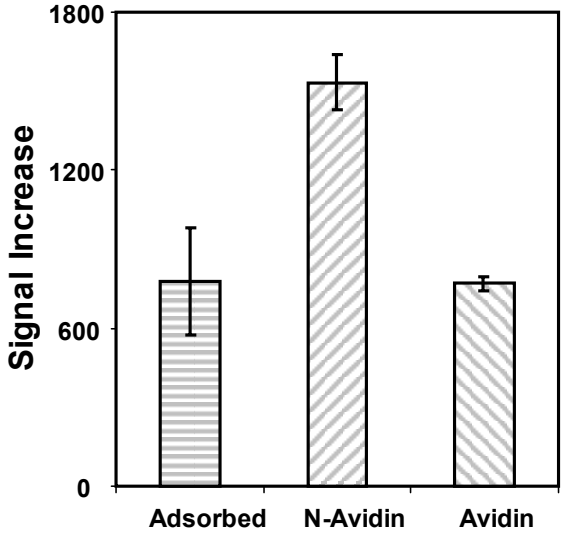


Figure 3. Comparison of capture antibody immobilization methods. An anti-*F. tularensis* antibody Mab-T-14 adsorbed or biotinylated and bound via avidin or NeutraAvidin. Signal due 10^7 cfu/mL and tracer regent, 10 ug/mL Alexa Fluor-labeled Mab-T-14, is compared

Two types of data are analyzed for each assay: assay rate and wash delta. The assay rate is the rate at which the fluorescent signal increases during the 90-second incubation with the tracer antibodies. The wash delta (pAmp) is calculated as the difference in signal at the completion of the assay from the signal obtained with the previous sample or background. The values obtained from both of these calculations are compared with pre-defined thresholds for determination of a positive or negative result. The names of the agents being tested, and whether the results are positive or negative, are displayed for each channel on the system's LCD upon completion of the assay. Although the use of both parameters greatly reduces the risk of false positives,[6] only the wash delta (signal increase) will be presented here for clarity.

RESULTS

With Research International's introduction of this most recent version of the RAPTOR, the Naval Research Laboratory took the opportunity to re-examine the biochemical options to improve assay signal strength, and thereby improve the ultimate limit of detection. The standard protocol for immobilization of the capture antibody onto the polystyrene optical probes had previously been simple adsorption. As an alternative to direct adsorption, we investigated the use of avidin to bind a biotinylated capture antibody, similar to the protocol we had adopted for use with silica optical fibers.[9] The first experiment utilized both avidin (pI = 10), and NeutrAvidin, a degylcosylated form of avidin that has a lower isoelectric point (pI = 6.3). Each was allowed to adsorb to the polystyrene optical probes at a concentration of

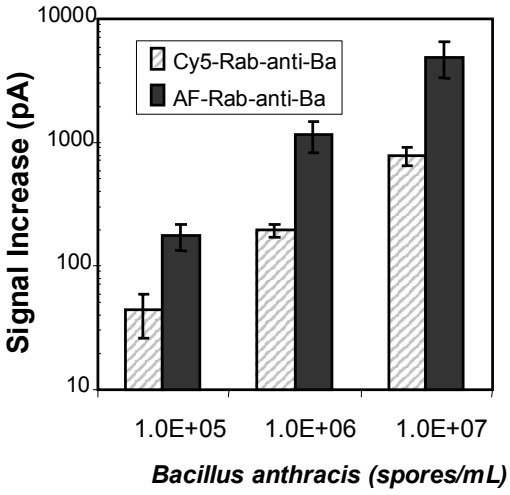


Figure 4. Comparison of different fluorescent dyes coupled to the tracer antibody reagent. Waveguides were coated with NeutrAvidin and biotinylated-affinity-rabbit anti-*Bacillus anthracis* IgG (100 μg/mL). Both Cy5- and AF 647-labeled affinity-rabbit anti-*Bacillus anthracis* IgG (20 μg/mL), having dye to protein ratios of 3 and 5.7 respectively, were used as fluorescent reagents. The signal generated by increasing concentrations of *Bacillus anthracis* is plotted

100 μg/mL overnight, the standard protocol for antibody adsorption. In an assay for *F. tularensis*, the use of NeutrAvidin was superior to avidin when used in conjuction with the same

biotinylated Mab-T-14 monoclonal antibody, and it also gave approximately twice the signal as directly adsorbed Mab-T-14 (Figure 3). Similar results have been observed for other immunoassays as well.

Another method investigated to improve signal generation was the use of an alternative fluorphore to Cy5, the previous dye of choice. Alexa Fluor 647, which has properties similar to Cy5, was tested. In earlier tests performed on the previous RAPTOR version, we found the new dye, Alexa Fluor 647, to be superior to Cy5.[10] This improvement resulted from the capacity to label antibodies at higher dye-to-protein ratios before the fluorescent dyes become nonfluorescent due to self-quenching. We confirmed this observation by comparing Alexa Fluor 647-labeled affinity rabbit anti-*Bacillus anthracis* to the same protein labeled with Cy5 on the current RAPTOR (Figure 4).

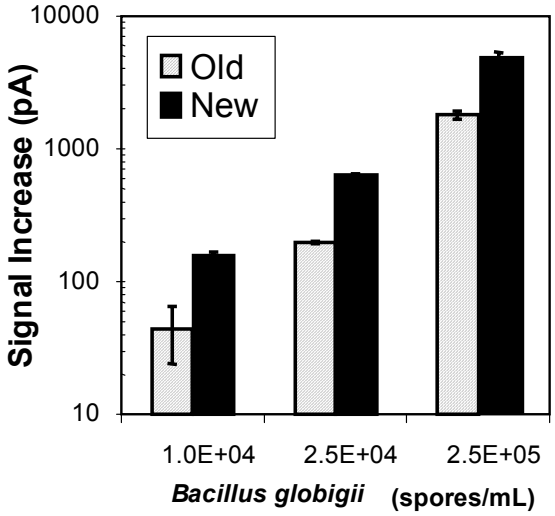
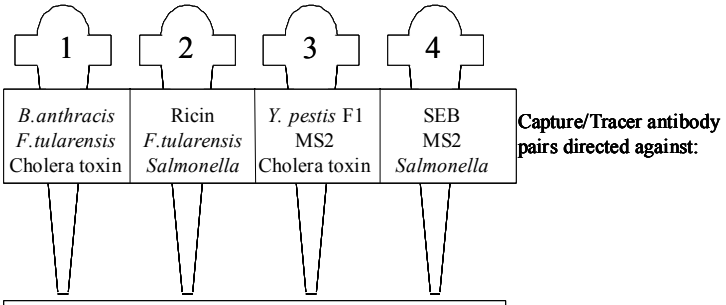


Figure 5. Comparison of the original and the most recently modified designs of the optical probes. A sandwich-assay was employed to detect *B. globigii* spores. The capture antibody used was biotin- rabbit anti-*B. globigii* (100 μg/mL) and the fluorescent tracer used was AF 647-rabbit anti-*B. globigii* (20 μg/mL).

One of the final system components to be optimized by Research International was the waveguide design. Numerous issues were refined to achieve a highly polished, optically clear, properly formed polystyrene waveguide. Early versions obtained were known to be sub-optimal. To verify that the "improvements" made to the waveguides actually translated to increased signal generation, we performed immunoassays for the detection of *B. globigii* spores (Figure 5). The newest version of the waveguides improved the signal by as much as 2- to 3-fold over the earlier molded waveguides. This signal increase translated directly into improvements in the RAPTOR's limit of detection.

Proof that the summation of all these improvements has yielded a system more sensitive by a factor of ten was subsequently shown during trials. This improvement was best exemplified by RAPTOR's improved ability to detect *B. anthracis* spores. During trials held in April of 2001, the RAPTOR detected only 1 of 16 samples of *B. anthracis* at 10^5 spores/mL, interspersed among 256 samples, while in June of 2002 the RAPTOR utilizing all the improvements described here detected 17 of 17 samples of *B. anthracis* at 10^5 spores/mL, interspersed among 132 samples. Work continues to enhance the RAPTOR's sensitivity even further.

A final advantage of the new RAPTOR is the ability to detect of any one of eight analytes in a sample utilizing a single coupon. This advance was the result of having separate tracer antibody vials for each waveguide. Using mixtures of both capture and tracer antibodies with a different combination of antibodies for each fiber makes each fiber specific for three different analytes. Then by using the response pattern of the waveguides one can detect and identify up to eight analytes in a single sample. A test of this arrangement, shown in Figure 6, has recently been demonstrated.[11]



Response Pattern Observed				Analyte
POS	---	---	---	*B. anthracis*
---	**POS**	---	---	Ricin
---	---	**POS**	---	*Y. pestis* F1
---	---	---	**POS**	SEB
POS	**POS**	---	---	*F. tularensis*
---	---	**POS**	**POS**	MS2
POS	---	**POS**	---	Cholera toxin
---	**POS**	---	**POS**	*Salmonella*

Figure 6: Response of mixed analyte RAPTOR assays for eight analytes. Each fiber is coated with a mixture of capture antibodies directed against three analytes; a complementary mix of tracers is used to interrogate the fibers after sample has been exposed. Results (positive/negative) are shown below each fiber. Refer to Lingerfelt et al. for experimental methods and detailed results.

CONCLUSION

This latest RAPTOR version has redesigned fluidics, which includes reliable peristaltic pumps, and redesigned optics, which include modified coupons and waveguides. In addition, we have established that the use of NeutrAvidin - biotin to attach capture antibody to the waveguide. These improvements when coupled with the improved fluorescent properties of Alexa Fluor 647 provide enhanced detection signals. In total, these improvements have produced a system that is not only easier and more reliable to operate but achieved a log-order of magnitude more sensitivity than previously described. We have demonstrated that improved sensitivity and the capability to detect a broad range of agents here with immunoassays for SEB, *F. tularensis, B. anthracis*, and *B. globigii* spores. In addition, we have also demonstrated the ability to detect and identify any one of eight different targets during a single analysis.

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